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# Histopathology and selective biomarker expression in human meibomian glands

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## ABSTRACT

**Background/aims** Meibomian gland dysfunction (MGD) is the most common form of evaporative dry eye disease, but its pathogenesis is poorly understood. This study examined the histopathological features of meibomian gland (MG) tissue from cadaver donors to identify potential pathogenic processes that underlie MGD in humans.

**Methods** Histological analyses was performed on the MGs in the tarsal plates dissected from four cadaver donors, two young and two old adults, including a 36-year-old female (36F) and three males aged 30, 63 and 64 years (30M, 63M and 64M).

**Results** The MGs of 36F displayed normal anatomy and structure, whereas the MGs of 30M showed severe ductal obstruction with mild distortion. The obstruction was caused by increased cytokeratin levels in association with hyperproliferation, but not hyperkeratinisation. In two older males, moderate to severe MG atrophy was noted. Cell proliferation was significantly reduced in the MG acini of the two older donors as measured by Ki67 labelling index ( $6.0\% \pm 3.4\%$  and  $7.9\% \pm 2.8\%$  in 63M and 64M, respectively) when compared with that of the two younger donors ( $23.2\% \pm 5.5\%$  and  $16.9\% \pm 4.8\%$  in 30M and 36F, respectively) ( $p < 0.001$ ). The expression patterns of meibocyte differentiation biomarkers were similar in the older and younger donors.

**Conclusion** Our histopathological study, based on a small sample size, suggests potentially distinct pathogenic mechanisms in MGD. In the young male adult, hyperproliferation and aberrant differentiation of the central ductal epithelia may lead to the obstruction by overproduced cytokeratins. In contrast, in older adults, decreased cell proliferation in acinar basal epithelia could be a contributing factor leading to MG glandular atrophy.

## INTRODUCTION

Meibomian glands (MGs), located in the upper and lower tarsal plates of the eyelids, are holocrine glands composed of secretory acini surrounding a central duct.<sup>1</sup> Based on their anatomical locations, acinar epithelia cells are classified as basal (germinative or proliferative), differentiating, mature and superficial degenerated cells. Each acinus at its basal circumference comprises a single layer of proliferative epithelial cells, which are essential for acinar regeneration. When the basal cells start to differentiate, they move inward and mature into lipid-producing meibocytes. After reaching maturity, the meibocytes disintegrate as holocrine cells and release lipids (meibum) into the short collecting

ductules that connect with the central secretory duct lined by the squamous epithelial cells. Meibum produced from the mature and decomposed meibocytes travels through the ductule to the central duct and is discharged through the central duct and onto the ocular surface via the MG orifice. The secreted meibum constitutes the outermost layer of the tear film, overlying the inner mucoaqueous layer and providing tear film stability.<sup>2</sup> Normal meibum from healthy MGs forms a barrier against tear film evaporation and protects the ocular surface from microbial and environmental insults such as dust and pollen.

Meibomian gland dysfunction (or disease; MGD) is a term broadly used to encompass various functional abnormalities of MGs.<sup>3–5</sup> Conventionally, MGD can be classified as MG ductal obstruction, MG hyposecretion and MG hypersecretion. MG ductal obstruction and hyposecretion are more common than MG hypersecretion, which is frequently associated with eyelid inflammation and occurs in rosacea, chalazion and allergy.<sup>6–8</sup> Animal models of MGD have shown that ductal hyperkeratinisation can result in ductal obstruction, meibum stasis, cystic dilation and, eventually, acinar atrophy and MG dropouts.<sup>9</sup> Keratinised materials, however, are not normally present in the ducts of human MGs.<sup>10</sup> Compromised meibum quality is another contributing factor to ductal obstruction.<sup>11</sup> Studies in both animals and humans suggest that acinar epithelial abnormalities, such as diminished renewal of acinar basal cells and/or impaired meibocyte differentiation, contribute to the pathogenesis of age-related MGD.<sup>12–14</sup> Androgen deficiency and medications, such as isotretinoin, are also thought to play a role in MGD.<sup>15–17</sup>

The high prevalence of MGD and its diverse pathology implicate that it is a multifactorial disease,<sup>18,19</sup> but the histopathological changes and underlying molecular mechanisms of MGD have not been extensively elucidated, which makes it clinically challenging to diagnose and manage MGD effectively. To gain a better understanding of the pathogenesis of MGD, we performed histopathological examinations and biomarker expression analyses on MG tissues of four cadaver donors, including two young adults and two older adults. Our current study implies that fundamentally different pathogenic mechanisms underlie MGD. Despite the limited sample size in this study, our findings unveiled previously poorly described pathogenic mechanisms of MGD and shed light on potential avenues for the development of



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mechanism-targeted diagnostic and therapeutic strategies for MGD.

## MATERIALS AND METHODS

### Acquisition of donor tarsal tissues

Fresh tarsal tissues from four cadaver donors were obtained from Mid-America Transplant (St. Louis, Missouri, USA). The donors were free of known systemic illness and included two young adults and two older adults: a 30-year-old male (30M), a 36-year-old female (36F), a 63-year-old male (63M) and a 64-year-old male (64M). The medical and ocular histories of the donors were deidentified prior to removal of the tarsal plates from the inner upper eyelids of the fresh cadavers. The removal of donor corneas and tarsal plates was performed by eye bank staff under the standard protocol for procurement of ocular tissues. The use of human tissues in research conformed to the provisions of the Declaration of Helsinki and was exempted by the Washington University Human Research Protection Office, St. Louis, Missouri, USA.

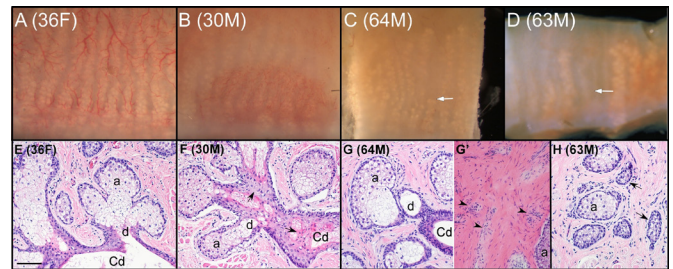
### Histology, immunofluorescence, immunohistochemistry and quantification of cell proliferation

Human tarsal tissues were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) and then processed for embedding in paraffin. Tissue sections (5 µm) were treated with xylene; rehydrated in a decreased ethanol series; and subjected to H&E staining, immunofluorescence or immunohistochemistry.<sup>20</sup> For antigen retrieval of immunostaining, tissue sections were boiled in 10 mM sodium citrate buffer for 10 min and then cooled to room temperature. To block endogenous peroxidase activity, sections were treated with 3% hydrogen peroxide in PBS for 20 min. Tissue sections were blocked with 3% horse serum in PBST (PBS plus 0.1% Tween-20) for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies diluted in the same buffer (table 1). Slides were washed with PBS, incubated at room temperature for 1 hour with either fluorophore-conjugated or biotinylated secondary antibodies and then washed with PBS. For immunofluorescence, cell nuclei were stained with 4',6-diamidino-2-phenylindole and slides were mounted with Mowiol (Sanofi-Aventis, Bridgewater, New Jersey, USA). For immunohistochemistry, sections were incubated with Vectastain Elite ABC Reagent (PK-6100; Vector Laboratories, Burlingame, California, USA) and colour was developed by using 3,3'-diaminobenzidine as a substrate

**Table 1** Sources and dilution ratios of primary antibodies

Protein	Company	Product catalogue no	Dilution ratio
FASN	Santa Cruz	sc-48357	1:500
Ki67	Cell Signaling	9129	1:500
Krt1	Santa Cruz	Sc-65999	1:1000
Krt6a	BioLegend	905 701	1:5000
Krt10	Covance (BioLegend)	PRB-159P	1:1000
Krt14	Covance	PRB-155P	1:1000
Krt16	Novusbio	NBP2-45538	1:1000
Krt17	Novusbio	NBP2-16089	1:1000
p63	Santa Cruz	SC -25268	1:200
PCNA	Abcam Inc.	ab29	1:1000
PPAR $\gamma$	Cell Signaling	2435	1:200

FASN, fatty acid synthase; PCNA, proliferating cell nuclear antigen; PPAR $\gamma$ , peroxisome proliferator activated receptor gamma.



**Figure 1** Human MG in tarsal plate and histology in MG tissue from four cadaver donors. (A–D) Tarsal plates dissected from cadavers' eyelids, designated as 36F (A), 30M (B), 64M (C) and 63M (D), were photographed directly before trimming off some of the orbicularis oculi muscle. Note: The thickness of the dissected tissue and setting of the photographic illumination gave rise to different appearance in tissue transparency. The two older donors (C, D) showed moderate to severe MG atrophy with glandular shortening (arrow in C) or dropout (arrow in D). (E–H) Histology (H&E staining). The circumference of MG acinus (a) in the 36F is made of a layer of basal epithelium that surrounds a cluster of lipid (meibum)-producing meibocytes (E). In 30M (F), thickened ductal epithelia (arrows in F) and hypercellularity were noted in the Cd. MGs of the two older donors (G and H) exhibited fibrotic areas with MG remnants (G', arrowheads) and acinar atrophy (H, arrows). Scale bar denotes 100 µm. 36F, 36-year-old female; 30M, 30-year-old male; 63M, 63-year-old male; 64M, 64-year-old male; a, acinus; Cd, central duct; d, ductule; MG, meibomian gland.

(D4293; Sigma-Aldrich, St. Louis, Missouri, USA). Sections were counterstained with haematoxylin for visualisation of cell nuclei.

Quantification of the cell proliferation was determined by Ki67 labelling index which is the ratio of Ki67-positive cells to the total basal epithelial cells counted, and the data were presented as means $\pm$ SD. Unpaired t-tests were used to compare the Ki67 index among individual donors or between the younger age group (30M and 36F) and the older age group (63M and 64M). A p value of less than 0.001 was considered statistically significant.

## RESULTS

### Histology of MGs in human cadaver donors

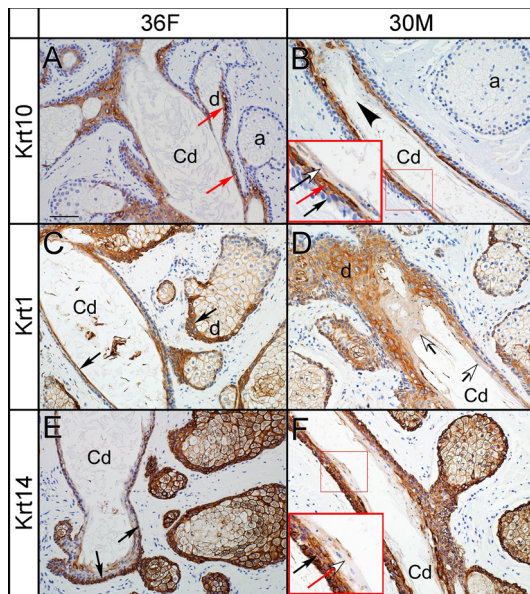
The MGs in the tarsal plates dissected from cadaver eyelids were first examined for gross morphology (figure 1A–D). The MGs of 36F were uniformly distributed in the tarsal plate and acini were evenly sized and appeared normal (figure 1A). Mild ductal distortion was observed in the MGs of 30M (figure 1B). In the two older donors (63M and 64M), MG atrophy, including glandular shortening and dropouts (arrows in figure 1C,D), was readily visualised in tarsal tissues.

Histology revealed notable variations in the acinar and ductal structure of MGs among the donors (figure 1E–H). In 36F, the lipid-producing acini were connected to the central duct by short branching ductules (figure 1E). By comparison, in 30M the MG central ducts were thickened from epithelial hypercellularity and filled with cellular debris and nuclear fragments (figure 1F, arrows). Compared with the two younger donors (30M and 36F), the MG tissues of the two older donors (64M and 63M) displayed altered features, with obliterated (figure 1G') and shrunken acini (figure 1H) scattered among areas containing normal-appearing acini (figure 1G).

### Ductal obstruction without increased keratinisation biomarkers

Krt10 and its type II pair Krt1 are considered to be keratinisation markers because they are expressed in terminally differentiated





**Figure 2** Expression of Krt10, Krt1 and Krt14 in MGs of the two young donors, one with normal-appearing MGs (36F) and the other with evidence of ductal obstruction (30M). (A,B) Krt10 was expressed in the suprabasal layers of ductules and central duct in 36F (A, red arrows). In the central duct of 30M, the Krt10-positive suprabasal layers (inset in B, red arrow) was 'sandwiched' between two Krt10-negative layers, presumably the basal layer (black arrow) and the superficial layer (open arrow). Ductal blockage (arrowhead in B) was caused by Krt10-negative proteins mixed with cell nuclear fragments. (C, D) Krt1 expression level in the MGs of 36F and 30M was high in the ductules and reduced in the suprabasal layer of the central duct (as indicated by arrows in C). Similar to the Krt10 pattern seen in MGs of 30M (B), the superficial layer of the central duct in this donor was negative for Krt1 (open arrows in D). (E, F) Krt14 was highly expressed in the MG acinar and ductule epithelia in both 36F (E) and 30M (F). Krt14 expression was limited to the basal epithelium in the central duct in 36F (arrows in E). The thickness of the Krt14-positive layer was notably increased in the central duct of 30M (F, black arrow in the inset). Scale bar denotes 100  $\mu$ m. 36F, 36-year-old female; 30M, 30-year-old male; a, acinus; Cd, central duct; d, ductule; MG, meibomian gland.

keratinocytes.<sup>21</sup> To investigate whether hyperkeratinisation of the ductal epithelia may account for the ductal obstruction in 30M, the expression patterns of Krt10 and Krt1 in MGs of the four donors were examined. We first verified the respective specificity of anti-Krt10 and anti-Krt1 antibodies by confirming their positive staining patterns at the mucocutaneous junction (MCJ) of cadaver eyelids (online supplementary figure S1). Expression of both Krt10 and Krt1 was interrupted at the MCJ where the epidermal epithelium transitions into the conjunctival epithelium (indicated by open arrows in online supplementary figure S1). Krt10 and Krt1 expression patterns in MGs of 36F, 63M and 64M differed from those of 30M. In 36F, both Krt10 and Krt1 were present in the suprabasal layer of the collecting ductules and the central duct (arrows in figure 2A,C). Similar patterns were seen in the MGs of 64M and 63M (data not shown). Interestingly, in the central duct of 30M, a Krt10/Krt1-positive suprabasal layer (red arrow in figure 2B inset) was sandwiched between the basal layer (black arrow) and a yet uncharacterised Krt10/Krt1-negative superficial layer (figure 2D, open arrows). The Krt10/Krt1-positive middle layer in the central duct of 30M was not thicker than the corresponding layer in the central duct

of the 36F. Furthermore, the debris blocking the central duct of 30M did not stain positive for Krt10 (figure 2B, arrowhead). Therefore, the Krt10/Krt1 expression pattern in 30M suggests that hyperkeratinisation is unlikely to be the direct cause of ductal obstructive MGD in this case.

The abnormal Krt10/Krt1 expression pattern in the central duct of 30M prompted us to investigate the expression of Krt14, a marker for basal cells in most epithelial tissues, including the skin.<sup>21</sup> In MGs of both 30M and 36F, Krt14 was highly expressed in the acini and in the basal epithelium of the central duct (figure 2E,F). However, in 30M, Krt14 expression was found not only in the basal layer of the central duct but also in multiple suprabasal layers (figure 2F inset). The abnormal and expanded expression of Krt14 in the central ductal epithelia was confirmed on multiple MGs from 30M and also by using cross sections of MGs from this donor (data not shown). These results suggest abnormal proliferation and differentiation of the central ductal epithelia may cause ductal obstruction in this donor.

### Ductal obstruction from overproduction of cytokeratins linked to hyperproliferation

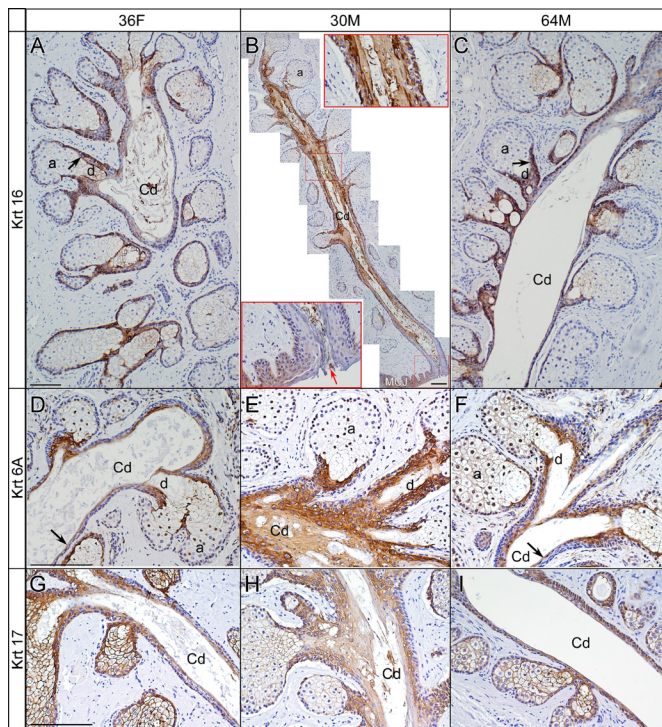
Keratin 16 (Krt16) is typically expressed in stratified squamous epithelia and certain glandular tissues.<sup>20–22</sup> Overexpression of Krt16 and its type II pair Krt6, along with Krt17 overexpression, is associated with keratinocyte hyperproliferation and considered to be hallmarks for psoriasis, a chronic inflammatory skin disease.<sup>23–24</sup> To explore the role of these cytokeratins in MGD, we examined the expression of keratins 16, 6a and 17 in the MGs of 30M, 36F and 64M (figure 3).

In MGs of 36F and 64M, Krt16 was mostly expressed in the ductules adjacent to the central duct, moderately expressed in the acinar periphery and minimally expressed in the MG central duct (figure 3A,C). In contrast, in the MG of 30M, Krt16 was overexpressed in the central duct (figure 3B, top right inset) and a 'plug', which was not stained by Krt16, was present in the orifice (figure 3B, red arrow in bottom left inset). Similar to the expression of Krt16, Krt6a expression in the MGs of 36F and 64M was greatest in the ductules adjacent to the central duct but attenuated in the central duct (figure 3D,F). In contrast, Krt6a was overexpressed in the central ducts of 30M (figure 3E), consistent with the aforementioned Krt16 overexpression in this young male donor.

Like Krt16 in psoriatic dermatitis, overexpression of Krt17 has been found in this disease.<sup>25</sup> In our study of MGs, Krt17 was distributed in acinar and ductal epithelia more ubiquitously than Krt16/Krt6a (figure 3G–I). The Krt17 level appeared to be increased in the thickened central ducts of 30M, as compared with Krt17 expression in the central ducts of 36F and 63M (compare figure 3H with figure 3G,I). Taken together, the findings suggest that overproduction of Krt16/Krt6a and Krt17 in the central ductal epithelia of the 30M is likely the underlying or a contributing cause of ductal obstruction in this younger male donor, who probably had clinically undetected obstructive MGD.

### Hyperproliferation and aberrant differentiation of central ductal epithelia

Because overexpression of Krt16 and Krt17 is associated with keratinocyte hyperproliferation, we examined the cell proliferation markers Ki67 and proliferating cell nuclear antigen (PCNA) in central ductal epithelia and compared the levels in 30M and 36F (figure 4A–D). Both Ki67- and PCNA-positive cells were markedly increased in the basal epithelia of the central duct of 30M when compared with the levels in the central duct of 36F. Furthermore, the Ki67 labelling index in the MG ductal basal epithelia of 30M

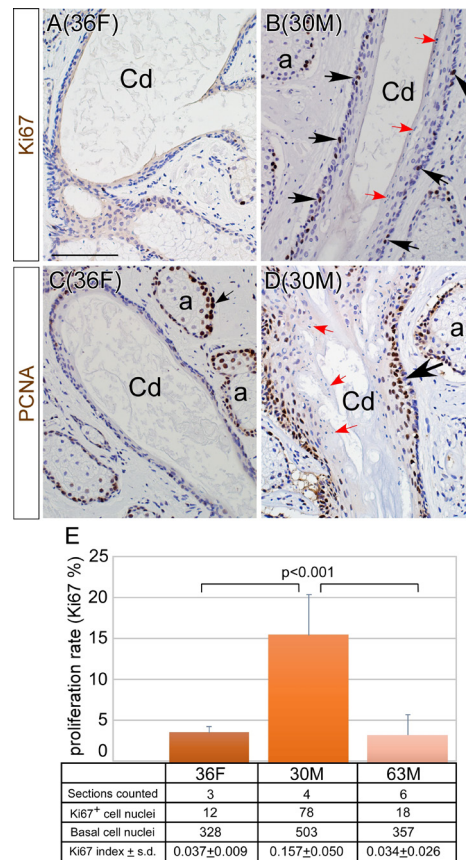


**Figure 3** Expression of Krt16, Krt6a and Krt17 in MGs of an older donor (64M) and of the two young donors, one with normal-appearing MGs (36F) and the other with evidence of ductal obstruction (30M). (A–C) Krt16 protein was mostly distributed in MG ductules and decreased in the MG central duct of 36F (A) and 64M (C) as compared with Krt16 expression in 30M (B). The central ductal cells in 30M showed Krt16 overexpression (B, montage and inset on the top right) and an orifice ‘plug’ (B, red arrow in bottom left inset). (D–F) Krt6a expression was similar to Krt16 expression in the MG ductules of 36F (D) and 64M (F). Krt6a overproduction was seen in the central duct of 30M (E). (G–I) Krt17 was ubiquitously expressed in the MGs of 30M, 36F and 64M, with a higher level in the epithelial cells of ductules. Krt17 overexpression was noted in the central duct of 30M, but not in the central duct of 36F and 64M (H). Scale bar denotes 100  $\mu$ m. 36F, 36-year-old female; 30M, 30-year-old male; 64M, 64-year old male; a, acinus; Cd, central duct; d, ductule; MG, meibomian gland.

was higher than that of 36F and 64M ( $p < 0.001$ ) (figure 4E). The histopathology also showed that nuclear fragments were retained in the thickened suprabasal layer of the central duct of 30M (red arrows in figure 4B,D), suggesting the abnormal differentiation of the ductal epithelia. These results together suggest that the potential cause of obstructive MGD in 30M is epithelial hyperproliferation with aberrant differentiation, associated with overproduction of cytokeratins, leading to the accumulation of proteins and nuclear debris in the central duct.

#### Age-related decline of acinar cell renewal and gland atrophy

Transcription factor p63 (TP63), a member of p53 family, is expressed by proliferative stem cells or progenitor cells in several stratified epithelia.<sup>26 27</sup> We found p63 expression in the basal epithelia of MG acini, the ductule and the central duct in MG tissue of each donor (figure 5A,B and data not shown for 30M and 64M), but p63 expression was reduced in some of the MG basal acini of 63M (figure 5B, red ‘a’). When cell proliferation marker Ki67 was colocalised with p63, Ki67-positive cells were also notably decreased in the acini of 63M, as compared with the number of Ki67-positive cells in the acini of 36F (compare

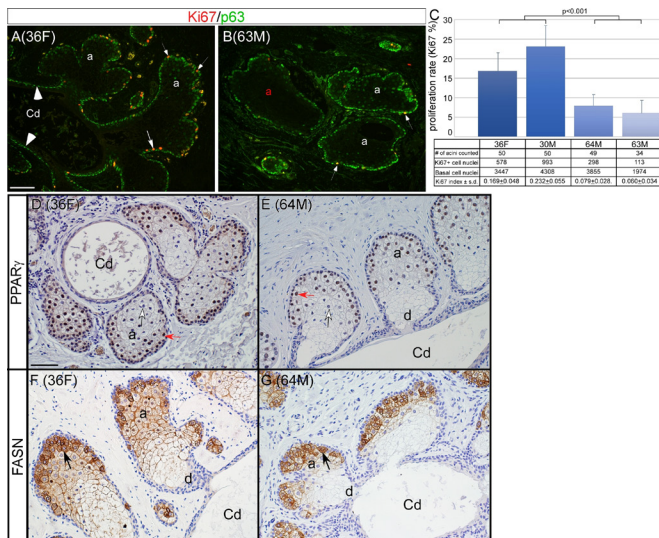


**Figure 4** Expression of cell proliferation markers in MGs of the donor with ductal obstruction (30M), the donor with normal-appearing MGs (36F) and one of the older donors (63M). (A, B) Ki67 expression. Ki67-positive cells were rarely identified in the basal epithelia of the central ducts in 36F (A). In contrast, many Ki67-positive cells were noted in the basal epithelia of 30M (B, black arrows). (C, D) PCNA expression was found in the nuclei of the acinar basal epithelium of 36F (C, arrow), and expression was diminished in cells differentiating into meibocytes. PCNA-expressing cells were rarely detected in the MG central duct in 36F (C), whereas in 30M PCNA-positive cells were abundant in the epithelia of the central duct (D, black arrow). In 30M, cell nuclear fragments were retained in the superficial layer of the MG central duct (B and D, red arrows). (E) Ki67 labelling index in MG ductal basal epithelia. Cell proliferation rate in ductal basal epithelia was higher in 30M than in 36F and 63M ( $p < 0.001$ ). Scale bar denotes 100  $\mu$ m. 36F, 36-year-old female; 30M, 30-year-old male; 63M, 63-year old male; a, acinus; Cd, central duct; D, ductile; MG, meibomian gland; PCNA, proliferating cell nuclear antigen.

figure 5B with figure 5A). The Ki67 labelling index was significantly lower in the older group ( $6.0\% \pm 3.4\%$  and  $7.9\% \pm 2.8\%$  for 63M and 64M, respectively) than that in the younger group ( $23.2\% \pm 5.5\%$  and  $16.9\% \pm 4.8\%$  for 30M and 36F, respectively) ( $p < 0.001$ ) (figure 5C), suggesting that this decrease of cell proliferation may compromise acinar cell renewal and haemostasis in older individuals, leading to age-related MG atrophy (figure 1D).

The main function of MGs is to produce and secrete lipids (meibum) that coat the outer tear film and prevent its evaporation.<sup>28</sup> The expression patterns of the meibocyte differentiation markers, peroxisome proliferator activated receptor gamma and fatty acid synthase, were compared between the 36F and older (64M) donor, representing a young with normal-appearing





**Figure 5** Expression of cell proliferation and differentiation biomarkers in MG acini of the 30-year-old, 36-year-old, 63-year-old and 64-year-old donors. (A, B) p63 (green nuclear staining) was expressed in acinar (a) and ductal basal epithelia in the MGs of 36F (A) and 63M (B). Notably fewer Ki67-positive cells (red nuclear staining pointed by white arrows) were present in Mg acini of 63M acini (B), when compared with those in 36F's acini (A). Reduction of p63 expression was noted in 63M (marked with red 'a' in B). (C) Bar graph depicting the Ki67 labelling index in MG acinar basal epithelia. The proliferation rate in basal acini was significantly higher in two younger donors (36F and 30M) than in the two older donors (64M and 63M) ( $p < 0.001$ ). (D–G) PPAR $\gamma$  expression (D and E, red arrows) and FASN expression (F and G, black arrows) were relatively intense in newly differentiated meibocytes and attenuated to background level in mature meibocytes (D and E, open arrows) in both 36F and 64M. Scale bar denotes 100  $\mu$ m. 36F, 36-year-old female; 30M, 30-year-old male; 63M, 63-year old male; 64M, 64-year old male; a, acinus; Cd, central duct; d, ductile; FASN, fatty acid synthase; MG, meibomian gland; PPAR $\gamma$ , peroxisome proliferator activated receptor gamma.

MGs and an older donor with age-related atrophy. The staining patterns for these differentiation biomarkers were similar in the MGs of 36F and 64M (figure 5D–G), suggesting that, in this older donor's acini where cell proliferation was reduced (figure 5C), the expression of meibocyte differentiation markers appeared to be normal.

## DISCUSSION

MGD is the major cause of evaporative dry eye disease and has diverse aetiologies.<sup>3 18 29</sup> Ductal obstruction and acinar or glandular atrophy are common features of MGD. To understand the pathogenesis of MGD, it is of paramount importance to investigate the histopathological changes in human MGs. This study describes the histological findings and the results of immunostaining for selective biomarkers in the MGs of cadaver tarsal plate tissues from two donors in the fourth decade of life (young age group) and two donors in the seventh decade of life (older age group). Despite of limited sample size, the data shed light on the notion that multiple pathogenic factors can contribute to the development of MGD.

Hyperkeratinisation of ductal epithelia has been shown in animal models of MGD to alter the structure and function of MGs by plugging the MG central ducts, causing ductal dilation and disruption of meibum excretion.<sup>9 10</sup> Ductal plugs comprising

non-keratinised epithelial cells have also been observed in animal models of MGD.<sup>12 30</sup> In this current study of human MGs, the tissue debris blocking the central ducts of 30M did not display the typical characteristics of hyperkeratinisation (figures 2 and 3). In the MGs of this donor, the keratinisation biomarkers Krt10 and Krt1 were present in the middle layer, above the Krt14-expressing basal layer, but underneath the superficial layer of the central ductal epithelia. Based on this abnormal expression pattern of the keratinisation biomarkers (Krt10 and Krt1), an alternative mechanism other than hyperkeratinisation seems likely to be the cause of ductal obstruction in this young male adult.

We propose that hyperproliferation and aberrant differentiation of the central ductal epithelial cells led to MG ductal obstruction and plugging in 30M. This conclusion is supported by the presence of overproduction of Krt6a/Krt16/Krt17 (figure 3) and increased cell proliferation markers (figure 4). Besides ductal obstruction, excessive cytokeratins are also disruptive to the quality of meibum as well as the stability of the lipid layer in the tear film. Meibum undergoes a maturation process as it is secreted into the ductules, with protein removal and/or lipid accumulation prior to excretion onto the ocular surface and incorporation into the tear film.<sup>31</sup> It has been shown that the addition of purified cytokeratins to meibum lipid can affect the fluidity of the meibum.<sup>32</sup> Such an increase in the protein–lipid ratio correlates with increased meibum viscosity.<sup>33</sup> Thus, it is conceivable that overproduction and retention of Krt6a/Krt16/Krt17 in the central duct, as found in the MGs of 30M, could have a profound effect on meibum quality and fluidity and lead to MG obstruction and the eventual development of MGD.

MGD is often associated with certain skin diseases, such as acne rosacea, atopic dermatitis, seborrhoeic dermatitis and psoriasis.<sup>6</sup> It was reported that patients with psoriasis vulgaris have a high prevalence of obstructive MGD, characterised by ductal plugging and impaired meibum secretion.<sup>34</sup> In psoriatic skin, the Krt6/Krt16 pair and Krt17, along with cell proliferation marker Ki67, are considered to be biomarkers for keratinocyte hyperproliferation.<sup>23</sup> The pertinent medical record of the 30M prior to corneal and tarsal plate removal did not reveal any indication of psoriatic skin lesions. Krt6 and Krt16 are known as stress-activated keratins.<sup>35</sup> Our interesting findings in this donor's MGs imply a potential utility of Krt6a/Krt16 staining of meibum excreta as diagnostic biomarkers to ascertain the diagnosis of obstructive MGD.

It is well documented that the process of ageing is associated with a decrease in the number of MGs along with an increase in MG dropouts.<sup>5 10 14 15</sup> Postulated factors include age-mediated hormonal changes, stem cell attenuation and growth factor deficiency. Other studies have demonstrated reduced Ki67 nuclear staining in old murine and human MGs,<sup>5 14</sup> which is consistent with our current finding of a reduced number of Ki67-positive cells in the acinar basal epithelia in MGs of 64M and 63M (figure 5C). Furthermore, we found that p63, a transcription factor for epithelial morphogenesis and stemness, was also downregulated in some acini in the MGs of 63M (figure 5B). Taken together, these observations suggest that a deficiency in acinar basal cell proliferation and/or MG progenitor/stem cell renewal may be a major contributing factor for the development of age-related MGD. In searching for the endogenous regulators responsible for MG cell renewal and homeostasis, we recently found that fibroblast growth factor receptor 2 (FGFR2) is highly expressed in both murine and human MGs. Using an inducible conditional knockout mouse model, we have demonstrated that FGFR2 signalling plays an essential role in MG acinar

proliferation and renewal.<sup>20</sup> Nevertheless, the significance of FGFR2 and its ligands in human MG homeostasis awaits further investigations.

In summary, while our study is limited by the small sample size due to the poor availability of donor eyelids, the histopathological and biomarker analyses of MG tissues from a young donor with normal-appearing MGs, a young donor with ductal obstruction and two donors in their early 60s shed insights into the diverse underlying pathogenic mechanisms of MGD. The findings of our study suggest that ductal epithelial hyperproliferation and abnormal differentiation, without notable hyperkeratinisation, can lead to MG obstruction, as in the case of 30M, whereas a decline in acinar cell proliferation and renewal is more likely the underlying cause of age-related MG atrophy, as in the two older donors. The findings also suggest that overexpression of hyperproliferative keratins in meibum may serve as potential biomarkers to diagnose specific type of obstructive MGD.

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**Data availability statement** Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as supplementary information.

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